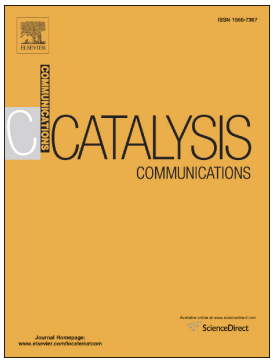

Discovery and application of methionine sulfoxide reductase B for preparation of (S)-sulfoxides through kinetic resolution

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Discovery and application of methionine sulfoxide reductase B for preparation of (*S*)-sulfoxides through kinetic resolution

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Abstract

Here we report a methionine sulfoxide reductase B (MsrB) enzymatic system for the preparation of (*S*)-sulfoxides through kinetic resolution of racemic (*rac*) sulfoxides. Eight MsrB homologue recombinant proteins were expressed and their activities on asymmetric reduction of *rac*-sulfoxides were analysed. Among these MsrB homologue proteins, one protein from *Acidovorax* species showed good activity and enantioselectivity towards several aryl-alkyl sulfoxides. The (*S*)-sulfoxides were prepared with 93-98% enantiomeric excess through kinetic resolution at initial substrate concentration up to 50 mM. The establishment of MsrB catalytic kinetic resolution system provides a new efficient green strategy for preparation of (*S*)-sulfoxides.

Keywords

Methionine sulfoxide reductase B; (*S*)-sulfoxides; Kinetic resolution; Biocatalysis

1. Introduction

Chiral sulfoxides are valuable organosulfur compounds and being used in a wide range of industrial fields [1, 2]. For instance, optically active sulfoxides are important in asymmetric synthesis, either as chiral building blocks or as stereogenic centers [3, 4]. Moreover, enantiopure sulfoxides have been widely used as pharmaceuticals in which usually only one of the enantiomers is responsible for the desired bioactivity [5, 6]. Thus, the enantioselective preparation of chiral sulfoxides is highly interested in both asymmetric synthesis and pharmaceutical industry. Chiral sulfoxides could be obtained through chemical or biological methods [7, 8]. In the past few years, biocatalysis has been emerging as a valuable synthetic tool for the preparation of high added-value compounds, as it has many advantages including that enzymes can afford high efficiency and enantioselectivity under mild reaction conditions [9-11]. The general strategies for preparation of enantiopure sulfoxides include asymmetric oxidation of prochiral sulfides and kinetic resolution of racemic (*rac*) sulfoxides [7, 8]. Although the asymmetric oxidation is a more straightforward method [12-14], the biocatalytic kinetic resolution is becoming an effective approach for enantioselective preparation of chiral sulfoxides [15, 16]. In this approach, the unwanted sulfoxide enantiomer is transformed while leaving the desired enantiomer. Although the major drawback of kinetic resolution is the 50% of theoretical maximum yield, the development of reductase-oxidant biphasic reaction systems provide an alternative technique for overcoming this bottleneck [17].

In previous studies, several methionine sulfoxide reductase A enzymes have been discovered and applied for the preparation of (*R*)-sulfoxides through reductive

resolution of *rac*-sulfoxides [15, 16, 18]. A series of aryl-alkyl sulfoxides in *R* configuration were prepared with approximately 50% of yield and 99% enantiomeric excess (*ee*) at the substrate concentration up to 320 mM (approximately 45 g/L) [16]. The extremely high substrate tolerance and enantioselectivity indicates that reductive resolution would become an effective strategy for green synthesis of chiral (*R*)-sulfoxides. On the other hand, attempting for preparation of (*S*)-sulfoxides by reductive resolution has also been reported years ago [19-22]. For instance, there were some cases using bacterial dimethylsulfoxide (DMSO) reductase for preparation of the (*S*)-sulfoxides and showed good enatioselectivity and substrate scope [22]. Although the substrate concentrations in these examples was still far from industrial scale, these reports provide an alternative reductive resolution strategy for preparation of (*S*)-sulfoxides.

Methionine sulfoxide reductase (Msr) is a class of enzymes which reduce methionine sulfoxide (Met-O) to methionine (Met) in cells [23, 24]. These enzymes play an important role in protein repairing and protect cells from oxidative stress [25, 26]. There are three types of Msr proteins: MsrA which specifically reduces the (*S*)-enantiomer of Met-O; MsrB which specifically reduces the (*R*)-Met-O in peptide and fRMsr, of which there are two forms: one that reduces the free (*S*)-Met-(O) and one that reduces the free (*R*)-Met-O, and is also called fRMsr [27, 28]. Considering the high activity of MsrA shown in the preparation of (*R*)-sulfoxides through reductive resolution, we hypothesized that MsrB or fRMsr could be similarly exploited for the preparation of (*S*)-sulfoxides. Recently, Nosek and colleagues investigated several MsrB homologues for preparation of (*S*)-sulfoxides and concluded that the low activity of natural MsrBs prevented them from application for

kinetic resolution of sulfoxides [22]. However, the Msr enzymes belong to a superfamily of proteins widely expressed in living species [29, 30]. Therefore, there could be MsrB or fRMsr forms with higher specific activity. We thus tested the activities of several MsrB and fRMsr homologues aimed to obtain Msr enzymes for preparation of (*S*)-sulfoxides. Finally, an MsrB homologue with good activity and enantioselectivity was successfully obtained.

2. Experimental

2.1 General

Commercial chemicals were purchased from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China), or Sangon Biotech Co., Ltd.(Shanghai, China). All sulfoxides were purchased from TCI Co., Ltd. (Shanghai, China), and J&K Scientific Co., Ltd. (Beijing, China).

2.2 Screening, cloning and expressing of recombinant MsrB and fRMsr homologues

The ORF of MsrB and fRMsr from *Pseudomonas moneilii* (*pm*MsrB and *pm*fRMsr) were amplified by PCR using genomic DNA of *Pseudomonas monteilii* CCTCC (China Center for Type Culture Collection) M2013683 we isolated before as template [31]. The gene sequences have been submitted to the Genbank database (accession number: MN312201 for *pm*MsrB and MN312202 for *pm*fRMsr). The protein BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied to search MsrB homologues using protein sequence of *pm*MsrB as template. Seven MsrB protein

sequences with sequence identity from 55%-92% were selected randomly. After obtaining the corresponding nucleotide sequences by tblastn program, the DNA fragments of these MsrB genes were synthesized. Then, the DNA fragments were inserted into the multiple cloning site (MCS) of the pET-28a. To induce the soluble expression of recombinant proteins, the recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells. After overnight culturing, cells containing expression plasmid was diluted 1:100 into 1 L of LB medium (1% peptone, 0.5% yeast extract, and 1% NaCl) with 50 mg/L of kanamycin and grown at 37 ◦C until it reached an absorbance at 600 nm (A_{600}) of 0.6. Cells were then induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 14 h at 20 ◦C and harvested by centrifugation at 2810 g for 10 min. After freezing the cells at -20 ◦C over 1 h, lysis buffer (50 mM phosphate, 1 mg/mL lysozyme, and 5 μg/mL DNase, pH 7.0) was added and cells were incubated at room temperature for 1 h. The soluble total proteins were obtained by centrifuging for 15 min at 17000 g at 4 ◦C and defined as crude enzyme. Proteins were quantified by the method of Bradford and subjected to SDS-PAGE to check their expressions.

2.3 Relative activity assay

Relative activities of MsrB from *Acidovorax sp. KKS102* (*ak*MsrB) under different pH and temperature conditions were determined by a colorimetric assay according to our previous report [16]. Briefly, MsrB crude enzyme (0.5 mg/mL), DTT (1 mM), and racemic methyl phenyl sulfoxide (*rac*-**1a**,1 mM) were assembled in 1 mL reaction system. After 20 min of reaction, the mixture was diluted 20 times with 50 mM phosphate buffer (pH 8.0). Then, 100 μL diluted samples were transferred into a

96-well plate, and another 100 μL of reaction mixture (4 mM DTNB, 50mM phosphate buffer, pH 8.0) were added. The plate was incubated under 37 ◦C for 10 min, and a microplate reader was used to determine the absorbance at 415 nm (A_{415}). Proteins extracted from cells containing plasmid pET-28a was used as control. The decrease of A_{415} was used to describe the relative activities of *ak*MsrB.

2.4 Biocatalytic kinetic resolution of *rac*-sulfoxides

The process of kinetic resolution was based on our previously reported MsrA-DTT system [15]. Whole-cell containing recombinant MsrB protein was used as catalysts at the cell density of 30 g/L (fresh weight). DTT was added into the reaction buffer (50 mM phosphate) at the concentration of 2-fold of substrates. Biocatalytic reactions were performed at 5 mL of reaction volume with shaking at 300 rpm. After incubation, the reaction mixture was extracted twice with 5 mL of ethyl acetate each time with vigorous shaking. The organic layers were collected by centrifugation and then dried by anhydrous sodium sulfate. For separation and purification of sulfoxides **1a-1c** after reaction, 20-30 parallel reactions (20, 30, 10 mM of *rac*-**1a-1c**, respectively) were performed. After extracting and collecting the reaction mixture, the remaining sulfoxides **1a-1c** were purified by silica gel column chromatography with petroleum ether/ethyl acetate (3:1) as elution solvent.

2.5 Analytical methods

Quantification of **1a–1g** and **2a–2g** after reaction were determined by GC with Agilent 7820 GC (Agilent Technologies, Santa Clara, CA) using a HP-5 column (30

m×0.32 mm×0.25 μm) with an FID detector. DMSO was used as an internal standard. The *ee* value of **1g** was determined by GC using a CP-ChiraSil-DEX CB column (25 m×0.25 mm×0.25 mm). For other sulfoxides, *ee* was determined using a Shimadzu Prominence HPLC on a DaicelTM AD-H or OD-H chiral column (250×4.6 mm, 5μm) with UV detection at 254 nm. The percent *ee* was calculated by the equation: %*ee*=[([*R*] -[*S*])/([*R*]+[*S*])]×100%. The quantification of two enantiomers of residual **1b** after different reaction time was determined by HPLC on OD-H chiral column. Calculation of *E* value for enantioselectivity was as follows: $E=\ln[(1-c)(1-ee)]/\ln[(1+c)(1+ee)]$, where *c* denoted the conversions. Optical rotation data of sulfoxides was determined by RUDOLPH research analytical Autopol I-AP. The absolute configuration of **1a-1g** were assigned by comparison of the rotation data and the retention time in HPLC with previous reports [12]. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker-300 (300/75 MHz) spectrometer using $CDCl_3$ as a solvent and TMS as an internal standard.

3. Results and Discussion

3.1 Identification of Msr proteins for asymmetric reduction of sulfoxide

In our previous reports, an MsrA protein from *Pseudomonas monteilii* CCTCC M2013683 strain was successfully used to reduce the (*S*)-sulfoxides for the preparation of (*R*)-sulfoxides [15, 32]. Considering there are other two types of Msr enzymes (MsrB and fRMsr) active on the (*R*)-sulfoxides *in vivo*, these two genes (named as *pm*MsrB and *pm*fRMsr) were cloned from this bacteria and recombinantly expressed in *E. coli*. After induction by IPTG, both *pm*MsrB and *pm*fRMsr

recombinant proteins were successfully expressed in soluble form (Figure 1A). Then, we used *rac*-**1a** as substrate to test their activities. The *pm*MsrB showed good activity and moderate enationselectivity towards the substrate. After 16 h of reaction, 60% of **1a** was transformed to the corresponding sulfide **2a**, and the (*S*)-**1a** with 41% of *ee* was obtained. In the meanwhile, the *pm*fRMsr was almost inactive on the substrate **1a** (Table 1). Thus, the MsrB protein was considered for our further researches on kinetic resolution of *rac*-sulfoxides.

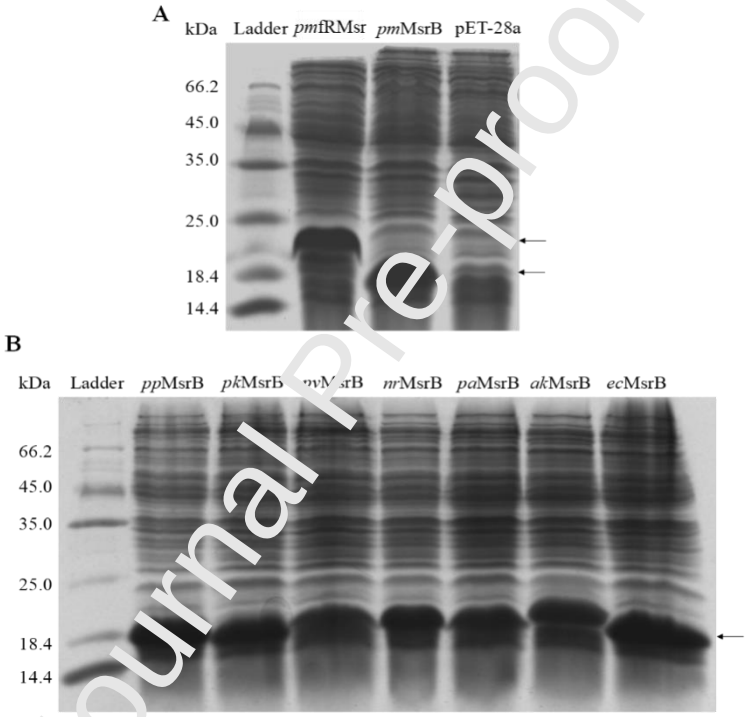
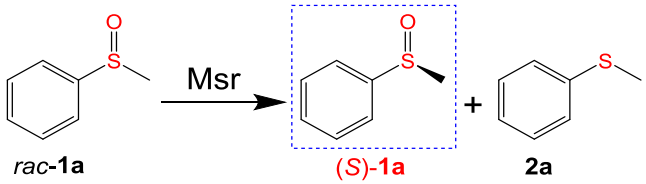


Figure 1 Recombinant expression of methionine sulfoxide reductase homologues. The soluble total proteins were subjected to SDS-PAGE gel electrophoresis. Arrows indicated the recombinant proteins. The names of proteins were corresponding to that in Table 2.

Table 1 Asymmetric reduction analysis of *rac*-**1a** by methionine sulfoxide reductases. (*t*=16 h, substrate concentration=2 mM)



Enzyme	Conversion(%) [a]	*ee* (%) [b]	*E* [c]
***pm*MsrB**	**60.5**	**41.4**	**3**
*pm*fRMsr	4.6	n.d.[d]	--
***pp*MsrB**	**58.0**	**91.6**	**15**
***pk*MsrB**	**47.3**	**57.2**	**8**
*pv*MsrB	22.7	8.0	2
*nr*MsrB	17.1	n.d.	--
*pa*MsrB	32.5	27.6	5
***ak*MsrB**	**60.8**	**96.0**	**16**
*ec*MsrB	20.4	n.d.	--

[a] Conversion of **1a** to the corresponding product **2a** was determined by GC after reaction.

[b] The *ee* value was determined by HPLC with OD-H chiral column.

[c] Calculation of *E* value was as follows: $E=\ln[(1-c)(1-ee)]/\ln[(1-c)(1+ee)]$, where *c* denotes the conversion.

[d] "n.d." means not determined due to low conversion.

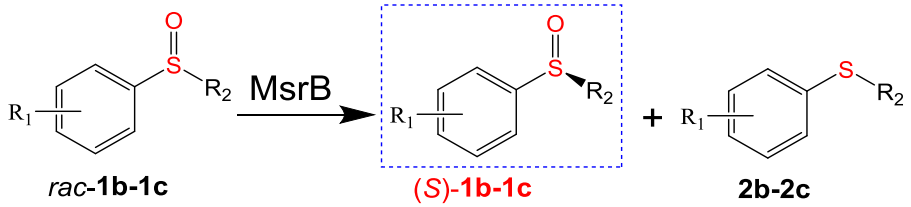
3.2 Screening of MsrB homologues for preparation of (*S*)-sulfoxides

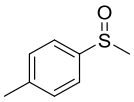
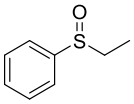
The MsrB belong to a superfamily of proteins widely expressed in living species [28]. Thus, we tested the activities of several *pm*MsrB homologues aimed to obtain MsrB enzymes with better catalytic properties. After sequence alignment using *pm*MsrB as template, seven *pm*MsrB homologues with sequence identities from 55%-92% were selected from GenBank database, and named as *pp*MsrB, *pk*MsrB, *pv*MsrB, *nr*MsrB, *pa*MsrB, *ak*MsrB and *ec*MsrB. The detailed protein sequences information was

summarized in Table S1. The sequence alignment of these proteins was carried out as shown in Supplementary Material (Figure S1). After induction, all seven recombinant proteins were successfully expressed (Figure 1B). The *rac*-**1a** was then used to test their activities and whole-cell containing recombinant Msr proteins were used as catalysts at the cell density of 30 g/L (fresh weight). The results showed that all these MsrB proteins were active on this substrate (Table 1). Among all tested MsrB enzymes, four proteins (*pm*MsrB, *pp*MsrB, *pk*MsrB and *ak*MsrB) were found to have good activity and enantioselectivity. After reaction by these four enzymes, 47.3%-60.8% of **1a** was transformed to the corresponding sulfide **2a**, and the (*S*)-**1a** with 41.4%-96.0% of *ee* was obtained (Table 1). In addition, considering that the expression of *pa*MsrB and *pv*MsrB was obviously lower than other enzymes (Figure 1B), we increased their cell densities from 30 g/L to 50g/L to test their activities. However, the conversions were only slightly improved (less than 10%), which indicates that the difference in expression level among these enzymes is not the key issue for their catalytic activities.

Therefore, we select these four MsrB proteins (*pp*MsrB, *pk*MsrB, *pa*MsrB and *ak*MsrB) to investigate their substrate specificity by testing their activity on several other aryl-alkyl sulfoxides **1b-1g**. Although the substrate structure affected their activity and enantiaoselectivity, all four enzymes did exhibited catalytic properties on these sulfoxides (Table 2 and S2), suggesting a certain level of compatibility for different sulfoxide substrates. Specifically, one MsrB protein from *Acidovorax sp.* KKS102 (named *ak*MsrB) showed high activity and enantiaoselectivity on the substrates *rac*-**1a-1c**, in which the (*R*)-**1a-1c** were almost completely transformed and (*S*)-**1a-1c** were obtained with *ee*>90% (Table 1 and 2).

Table 2 Kinetic resolution analysis of *rac*-**1b-1c** by 4 MsrB recombinant proteins. (t=16 h, substrate concentration=2 mM)



Substrate	Enzyme	Conversion (%)	*ee*(%)	*E*
 **1b**	*pm*MsrB	57.2	38.8	3
	*pp*MsrB	63.8	80.3	6
	*pk*MsrB	69.7	52.3	2
	***ak*MsrB**	**69.2**	**96.4**	**9**
 **1c**	*pm*MsrB	26.5	6.2	1
	*pp*MsrB	47.4	50.9	6
	*pk*MsrB	42.6	44.0	6
	***ak*MsrB**	**67.2**	**93.1**	**8**

3.3 Catalytic property analysis of *ak*MsrB

Considering that *ak*MsrB showed the best catalytic properties among these homologues, this enzyme was selected for further analysis. We firstly measured the relative activities of *ak*MsrB at temperatures ranging from 20 to 60°C. The maximum activity was observed at 35°C. Moreover, this enzyme exhibited good thermo-stability as more than 85% of activity retained at reaction temperature up to 50 °C. The effect of pH on the activity of *ak*MsrB was then tested with pH values ranging from 4.0 to 11.0. The results showed that this enzyme was sensitive to pH and the optimum pH was 7.0 (Figure 2). Taken together, pH 7.0 and 35°C were considered as the optimal reaction conditions for *ak*MsrB.

In the above reactions catalyzed by *ak*MsrB, we found that the conversions of *rac*-**1a-1c** to **2a-2c** were higher than 50%. These data mean that both the *R* and *S* enantiomers could be reduced by the *ak*MsrB whole-cell catalyst. To further understand the details of reaction, we chose *rac*-**1b** as substrate to investigate the time-dependent changes of the two enantiomers. The reactions were performed at a higher substrate concentration of 20 mM. After 6 h of reaction, over 80% of (*R*)-**1b** and 13.1% of (*S*)-**1b** were reduced to the sulfide (Figure 3). After 16 h of reaction, the (*R*)-**1b** was almost completely reduced (approximately 99%), while 17.3% of (*S*)-**1b** was also converted to the corresponding sulfide. These results indicate that the *ak*MsrB whole-cell catalyst could show activities both on (*S*) and (*R*)-sulfoxides, while the reduction of (*R*)-sulfoxide was much faster than (*S*)-sulfoxide. It is known that under stress conditions like stationary phase growth, *E. coli* cells upregulate sulfoxide reducing enzymes (*e.g.* MsrA) [33], which may thus diminish the selectivity factors. Therefore, we analyzed the catalytic activity of *E. coli* cells containing only pET-28a plasmid. As shown in Figure 3, 13.2% and 16.3% of (*S*)-**1b** was converted to the corresponding sulfide after 6 h and 16 h of reaction, respectively. These conversions were very close to that by cells containing *ak*MsrB enzymes. Thus, these results suggest that the activity of *ak*MsrB enzyme on (*S*)-sulfoxide probably very low and the real enationselcetivity could be much higher. In addition, the (*R*)-**1b** was only slightly converted (5.1% and 6.7% after 6 h and 16 h of reaction, respectively) by the cells containing pET-28a plasmid, which also proved that the reduction of (*R*)-**1b** was predominantly catalyzed by the recombinant enzyme other than the endogenous (*R*)-sulfoxide reductase of the cells. We further purified this recombinant enzyme and tried to test its enationselcetivity. Unfortunately, the activity of pure recombinant *ak*MsrB was extremely low and less than 10% of conversion was observed. Further

studies need to perform to investigate the mechanism and improve the stability of *ak*MsrB enzyme during purification and reaction.

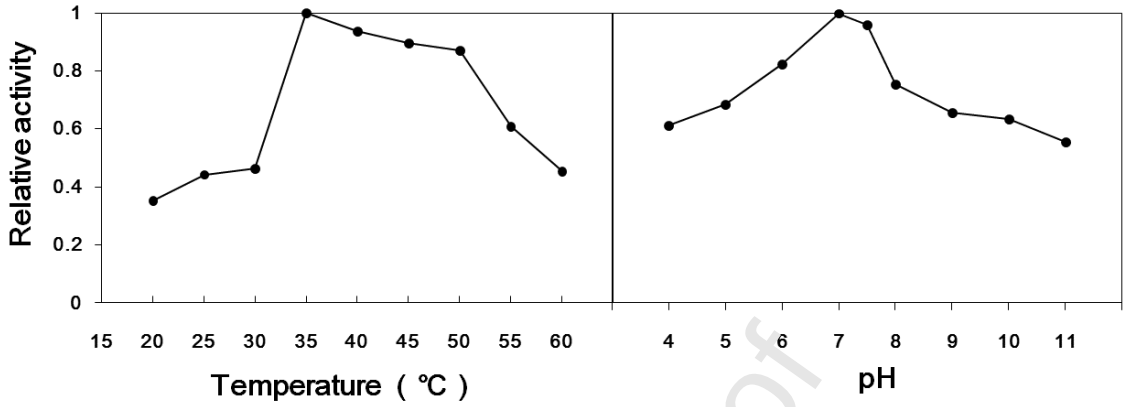


Figure 2 Optimization of temperature and pH for *ak*MsrB activity.

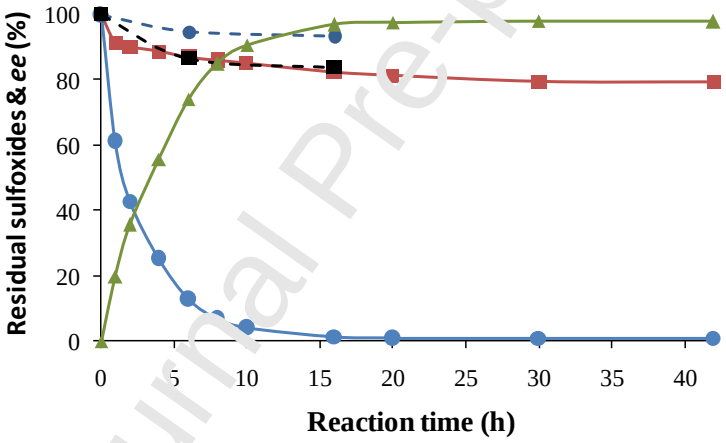


Figure 3 Analysis of residual sulfoxides in *R* (●) and *S* (■) configurations and the *ee* values (▲) of **1b** after reaction by whole-cell containing *ak*MsrB recombinant protein (solid lines) or only pET-28a plasmid (dashed lines) after different reaction time.

3.4 Substrate tolerance analysis of *ak*MsrB on kinetic resolution

The initial substrate concentration is an important issue in biocatalytic preparation of chiral sulfoxides, we thus investigated the substrate tolerance of *ak*MsrB whole-cell

catalyst. Concentrations of *rac*-**1a-1c** from 2 to 80 mM were used to test the enzyme tolerance to the substrates. The results demonstrated that the kinetic resolution of **1a-1c** was successfully accomplished with good enantioselectivities (E=13～53) at substrate concentrations up to 20, 50 and 10 mM, respectively. Under these substrate concentrations, the conversions of *rac*-**1a-1c** were 52.9%, 55.9% and 65.3%, and products (*S*)-**1a-1c** with 92.7%, 93.3% and 94.7% of *ee* were obtained, respectively (Figure 4). Compared to reported DMSO reductase system [19-22], our research represents a big improvement in the aspect of initial substrate concentration. More importantly, comparing to the membrane-associated multi-subunit DMSO reductase [22], soluble recombinant MsrB was much easier to be prepared. The simpler molecular structure of MsrB would exhibit great advantages in the subsequent process like protein engineering for further improvement of the catalytic properties. Finally, we performed these reactions of *rac*-**1a-1c** on preparative scale. The rotation data, ^{1}H and ^{13}C NMR spectra of purified residual **1a-1c** after reaction were determined and presented in Supplementary Material.

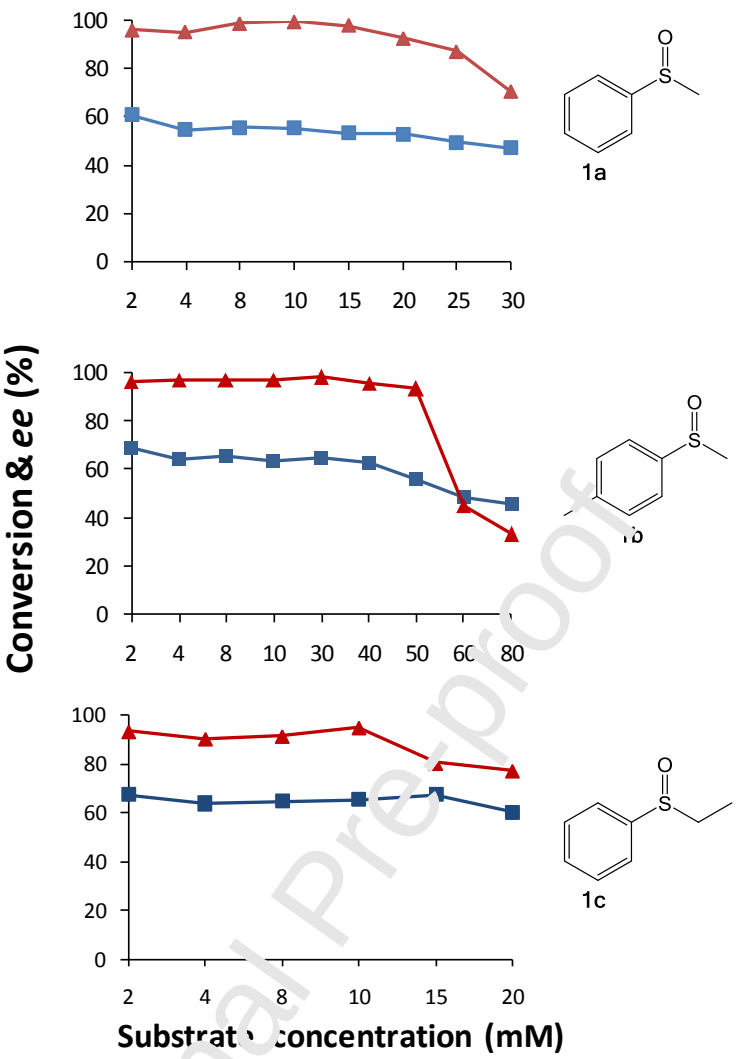


Figure 4 Conversion (■) and *ee* (▲) analysis of *rac*-**1a-1c** after reaction by *ak*MsrB at different substrate concentrations. *t*=16 h.

4. Conclusion

In summary, this study reports the first successful application of methionine sulfoxide reductase for the preparation of (*S*)-sulfoxides through kinetic resolution. The protein from *Acidovorax sp.* KKS102 (named *ak*MsrB) showed good activity on several *rac*-sulfoxides. The kinetic resolution of *rac*-sulfoxides was successfully accomplished at substrate concentrations up to 50 mM (7.8 g/L) when catalyzed by *ak*MsrB whole-cell catalyst. Our study supports that asymmetric reductive resolution

of *rac*-sulfoxides catalysed by MsrB could become an effective strategy for green synthesis of optically pure sulfoxides. Further engineering of these MsrB is ongoing to improve their catalytic performances, including substrate tolerance, substrate specificity and stability.

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Highlights

MsrB was firstly applied for (*S*)-sulfoxides preparation through kinetic resolution

MsrB from *Acidovorax* species showed good activity and enantioselectivity towards several *rac*-sulfoxides

(*S*)-sulfoxides were prepared with 93-98% of *ee* at substrate concentration up to 50 mM